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Molecular Epidemiology and Variation of Hepatitis B in Recent Immigrant Families to Australia

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Natural variation in hepatitis B virus (HBV) was studied in asymptomatic carriers originating from countries of high endemicity. HBV DNA was detected by dot blot and/or polymerase chain reaction (PCR) in 34 of the 184 members of 22 new immigrant families who agreed to be tested after one of their children had been found to have current or past hepatitis B in a school survey. PCR products from both the S and distal-X pre-C regions were sequenced. One vaccinated child had the classical ''escape'' mutation at amino acid position 126 in the S-gene and two other children and two adults had other substitutions at amino acid positions 133, 120, 165, and 159. Mutations were more frequent in the distal-X pre-C region and included two pre-C mutants and 13 other amino acid substitutions. The strains originating in the various countries were placed in almost identical groups by phylogenetic analysis using each amplicon, and determination of subtype by antigenic analysis gave the same result as sequencing. The S-data allowed recognition of three dominant strains within genotype B, while the distal-X pre-C data provided better discrimination between family groups. No change was found when the sequence of samples obtained for the study was compared with those collected from 14 of the children two years earlier. There was some evidence of horizontal spread in addition to vertical transmission. Reports of mutations of HBV in patients with severe or unusual clinical features should be interpreted with caution until the prevalence of the mutant in asymptomatic carriers has been determined. ***J. Med. Virol. 56:10–17, 1998.***

KEY WORDS: escape mutant; pre-C mutant; genotype; nucleotide substitutions; ethnic communities

INTRODUCTION

Social upheavals during the last 50 years have accelerated migration to Australia from countries in Southeast Asia and the Middle East, with medium to high rates of hepatitis B virus (HBV) carriage and a variety of antigenic subtypes. Studies in many countries, including the United States [Franks et al., 1990], Europe [Lindh et al., 1993], the Middle East [Sandler et al., 1978], as well as Australia [Kaldor et al., 1996], have shown that such immigrants retain the HBV infection pattern of their country of origin, at least in the first generation. The longer-term epidemiological consequences of immigration on HBV infection remain conjectural. On the one hand, environmental factors such as higher nutrition may increase the rate of viral clearance and better educational opportunities may delay the age of marriage and childbearing so that fewer pregnant women remain viremic during their childbearing years. On the other hand, sexual transmission between partners carrying different virus subtypes may facilitate viral recombination and the emergence of new strains with distinctive antigenic and biological properties. Universal vaccination in this environment may also encourage emergence of strains that lack the prototype "a" epitope [Carman et al., 1990; Fortuitin et al., 1994; Hino et al., 1995; Ngui et al., 1997].

In Australia, dispersion of diverse immigrant groups, carrying distinctive HBV strains within a predominantly low-prevalence community, offers an opportunity to observe the relative importance of virus strain, human genetic predisposition, and socioenvironmental factors in determining the established geographical differences in HBV prevalence, genotype, and disease load.

The present study builds on a survey [Burgess et al., 1993] of HBV in 2,883 primary schoolchildren in areas of Sydney with high immigrant populations. No evidence was found to suggest that transmission was occurring between children at school, but markers of current or past infection were present in 21% of the children belonging to immigrant families from high prevalence countries of Asia, the Pacific, and the Middle East.

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The natural variability of HBV has been determined in serum from a cohort of 32 schoolchildren with HBV identified in the school survey, and in all 152 other members of their families. We have compared the genomic sequences specifying the antigenic region of the S-gene and the distal-X pre-C region of these 72 HBV strains with data derived from studies in other countries. We also retested the 14 HBV-DNA-positive index children after a two-year interval in order to observe the rate of mutation of the virus.

MATERIALS AND METHODS

Subjects

A letter inviting the whole family to be tested for HBV was sent to the parents of all 51 HBsAg-positive children identified in a survey of over 2,883 11–12-year-old schoolchildren and also to 111 families of children with anti-HBc and anti-HBs as markers of past hepatitis B infection. Twenty-two of the families with an HBsAg-positive schoolchild and 11 of the other families agreed to participate. These families were visited at home for blood collection, which included a repeat sample from the index schoolchild, and administration of a simple questionnaire, which was available in appropriate translation. Physical examination was not carried out.

Liver Function Tests

Total and direct bilirubin, aspartate and alanine transferases, gamma glutaryl transpeptidase, and alkaline phosphatase were carried out on a Beckmann CX5 analyzer.

Hepatitis B Serology

HBsAg, and anti-HBs, and anti-HBc were carried out by radioimmunoassay (Abbott Laboratories) and HBe/antiHBe by ELISA (Abbott Laboratories).

Antigenic Subtyping

Samples from the family study were subtyped by gel diffusion using monoclonal antibodies according to the Paris classification [Couroucé et al., 1976].

HBV DNA

For dot blot hybridization [Dieguitis et al., 1986], 30 μl plasma volumes were pipetted into microtiter wells and mixed with equal volumes of 1 M sodium hydroxide. After 5 min agitation, 50 μl of the mixtures were transferred to corresponding wells of a dot blot manifold containing a prepared nylon membrane (Genescreen Plus, Dupont) After transfer and prehybridization at 65°C, the membrane was probed with ^{32}P-labeled pHBVCB [Burrell et al., 1979].

For PCR, 100 μl plasma samples were extracted with phenol and chloroform after proteinase K treatment and subjected to PCR using Tth Plus polymerase (Biotech International, Perth) and 40 cycles of amplification essentially after the method of Okamoto et al. [1992].

For amplification of 189 nt region of the S-gene, the 5′ and 3′ primers [McMahon et al., 1992] were 5′-TCGGATCCGGTATGTTGCCCGTTTGTCC-3′ and 5′-TCGAATTCGTGGCACTAGTAAACTGAGCC-3′, respectively. Amplification of a 258 bp sequence in the distal-X/pre-C region used forward and reverse primers 5′-TTCGCTTCACCTCTGCACGT-3′ and 5′-GAAGGAAAGAATTCAGAAGGCAAA-3′. The S-amplicon spans the "a" epitope and includes the nucleotides responsible for determining the d/y and w/r variation. The distal-X/pre-C amplican spans DR1 and epsilon motifs.

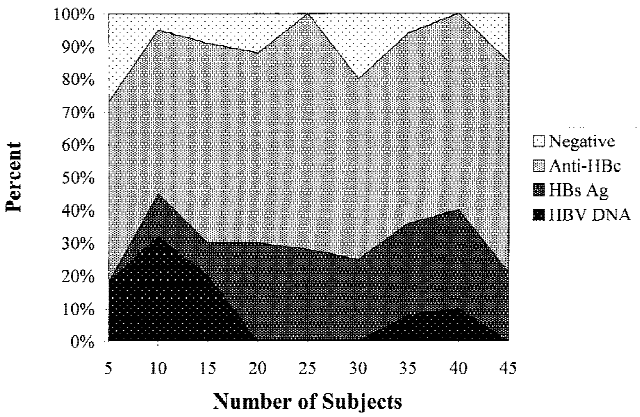

Fig. 1. Serological markers of hepatitis B infection in the study population, showing the very high level of current and past infection in all age groups. Viremia is present in a very high proportion of school-age children but is much less prevalent in young adults.

Sequencing

PCR products were purified by polyethylene glycol precipitation and sequenced by the dideoxy terminator method in an Applied Biosystems (Foster City, CA) model 373A DNA sequencer. Sequences were accepted for analysis only if data overlap was available in both directions. Alignment was performed using the Clustal W (1.4) multiple sequence alignment program [Higgins et al., 1992]. Acceptable sequence data was obtained for the S-amplicon between nt 497 and 664 and for the distal-X pre-C from nt 1643 to 1900. Dendrograms were constructed using the Kitch program [Felsenstein, 1993] and amino acid sequences using the translation program available in the Australian National Genome Information service (ANGIS).

RESULTS

There was a very high level of current and past infection in all the study families in Figure 1. Fourteen of the 21 HBsAg-positive index children aged 11–12 years (70%) were HBV-DNA-positive, the same proportion (24/33, 72%) as in adolescent family members aged 13–19. Adults over 25 years of age, however, had a much lower rate of active viremia (2/22, 10%). Only one of the 32 mothers and 2 of the 30 fathers whose children had markers of hepatitis B infection were viremic at the time of testing.

Of the 59 HBsAg-positive family samples tested, 29 were HBe-positive and 28 of these were HBV-DNA-

TABLE I. HBeAg Status, Antigenic Subtype, and Genotype in HBV-DNA-Positive School and Family Subjects[a]

Ethnicity	School survey number	Family/subject number	Sex/age	HBeAg status	Deduced subtype	Observed subtype	Genotype
Vietnamese	3493	1/1	M/12	positive	yw[1]1	yw[1]1	B
		2/14	M/14	positive	dw 2	dw 2	B
		2/13	M/13	positive	dw 2	dw 2	B
		3/125	F/44	negative	yw 1	ND	B
		4/68	M/44	negative	yw 1	weak	B
		4/71	F/17	positive	yw 1	yw 1	B
	2711	5/52	M/13	positive	dw 2	dw 2	B
		5/49	M/40	positive	dw 2	dw 2	B
		5/54	M/7	positive	dw 2	dw 2	B
		6/61	M/11	positive	yw 1	yw 1	B
	2429	7/92	M/13	positive	yw 1	weak	B
	2504	7/93	M/11	positive	yw 1	yw 1	B
		7/91	F/44	positive	yw 1	weak	B
		7/94	M/15	positive	yw 1	yw 1	B
		7/95	M/10	positive	yw 1	weak	B
		8/122	M/38	negative	yw 1	weak	B
		8/123	F/35	negative	yw 1	ND	B
	1827	10/132	M/12	positive	yw 1	yw 1	B
	2364	11/145	M/13	positive	dw 2	dw 2	B
		11/144	F/38	negative	dw 2	dw 2	B
		16/181	M/17	positive	yw 1	yw 1	B
	1862		M/12	positive	drq +	ND	C
	1906		M/11	positive	drq +	ND	C
	1948		M/12	positive	dw 2	ND	B
	1955		F/11	positive	dw 2	ND	B
	2182		M/11	positive	drq +	ND	C
	2395		F/11	positive	yw 1	ND	B
	3430		M/11	negative	yw 1	ND	B
	3605		M/12	positive	drq +	ND	C
Vietnamese/Cambodian		1/76	M/40	negative	dw 2	ND	B
	1930		M/11	positive	drq +	ND	C
Cambodian	2642	1/105	M/13	positive	yw 1	yw 1	B
	2266	2/82	M/13	positive	dw 2	dw 2	B
		2/78	M/44	positive	dw 2	weak	B
		2/77	F/18	positive	dw 2	dw 2	B
	3245	3/138	F/12	positive	drq +	dw 2	C
	1946		M/11	positive	drq +	ND	C
	2084		M/11	positive	drq +	ND	C
	2425		M/11	positive	drq +	ND	C
	3063		F/11	positive	drq +	ND	C
	3148		F/11	positive	drq +	ND	C
Cambodian/Thai	2087		M/11	positive	drq +	ND	C
	2090		F/11	positive	drq +	ND	C
	3072		M/11	positive	drq +	ND	C
Thai	3399	1/66	M/13	positive	drq +	drq$^+$	C
Laotin	2539	1/86	M/13	positive	drq +	drq$^+$	C
		1/87	M/15	positive	drq +	drq$^+$	C
		1/89	F/8	positive	drq +	drq$^+$	C
		3/101	M/44	negative	drq +	ND	C
	1928	4/111	M/13	positive	drq +	drq$^+$	C
	1924		F/11	positive	drq +	ND	C
	2729		M/11	positive	drq +	ND	C
Philippino	2215		F/13	positive	dw 2	dw 2	A
Maori	2267	1/37	M/12	positive	yw 1	yw 1	D
Tongan	2057		M/12	positive	drq –	ND	C
Lebanese		2/40	M/23	negative	yw 2	ND	D
Australian aboriginal	2747		F/11	positive	yw 2	ND	D
	3764		F/11	positive	yw 2	ND	D

[a]ND = no data.

positive by PCR and 23 by both PCR and dot blot. The remaining sample was negative by both PCR tests and gave a reading only slightly above the cutoff in the HBeAg ELISA. Eight of the HBe-negative, HBsAg-positive samples contained HBV DNA detectable by PCR, two of these were also positive by dot blot. Both were pre-C mutants with the classical TTG–TAG substitution at nt 1896.

The geographical origin of the 58 HBV-DNA-positive strains available for testing from the family study, together with the original school survey, was as follows: Vietnamese 29, Cambodian 10, Vietnamese/

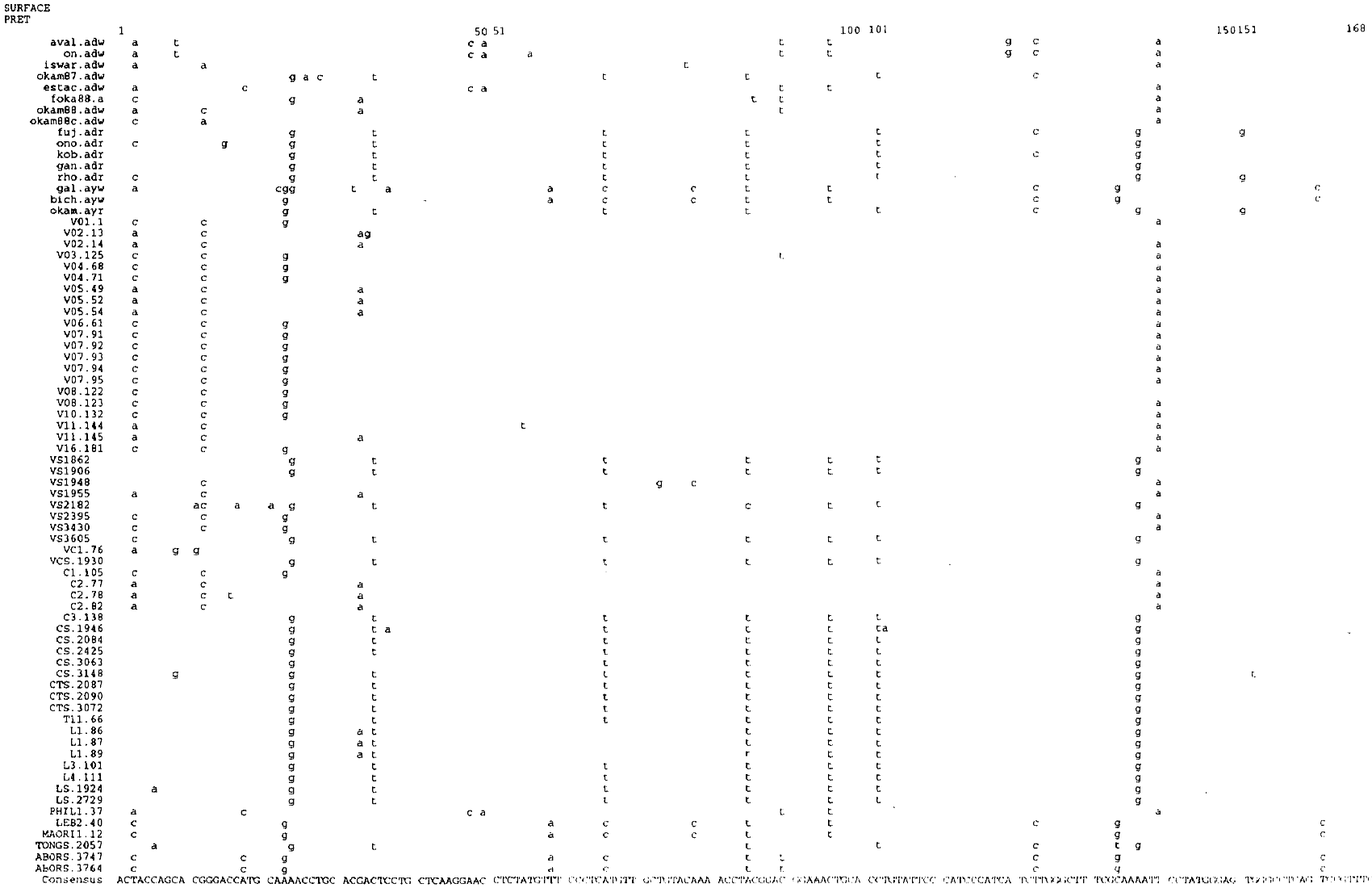

Fig. 2. Nucleotide sequences encoding amino acids 109–171 of the S-gene for the 58 HBV-DNA-positive study samples and 16 prototype HBV strains. The identification codes for the study strains are shown in Table I.

TABLE II. Amino Acid Substitutions in the S-Gene Among Asymptomatic Immigrant Family Members

Study number	Age/sex	HBe status	Amino acid position	Normal amino acid	Substitution	Vaccination
V2/13	14/M	positive	126	Thr (ACT)	Ala (GCT)	yes
V11/144	38/F	negative	133	Met (ATG)	Leu (TTG)	no
C2/78	44/M	positive	120	Pro (CCA)	Ser (TCA)	no
C3148	11/F	positive	165	Trp (TTG)	Leu (TTG)	no data
T2057	12/F	positive	159	Ala (GCA)	Val (GGT)	no data

Cambodian 2, Cambodian/Thai 3, Thai 1, Laotian 7, Philippino 1, Lebanese 6, Maori 1, Tongan 1, and Australian aboriginan 2. Two families had one parent from Vietnam and one from Cambodia, and three families had one parent from Cambodia and one from Thailand.

Subtypes were assigned to these strains from the sequence data for the S-gene as described by Okamoto et al. [1988] and Norder et al. [1992] and are shown in Table I, along with the antigenic subtypes determined by serology and the genotype assigned according to the criteria of Norder et al. [1992]. There was only one discrepancy between subtype deduced from sequence and the observed phenotype. However, six strains could not be serotyped, although they gave similar counts to typable strains in the HBsAg radioimmunoassay.

Apart from the recognized subtype determinants, 24 other nucleotide changes were observed in the S-gene sequences (Fig. 2). Nineteen of these produced no amino acid substitution in either the S or P open reading frame. The changes found in the remaining five strains are shown in Table II. Two strains had changes within the "a" determinant. One, Vietnamese strain 2/13 from a 14-year-old boy, demonstrated the putative escape mutant substitution of alanine for threonine at amino acid 126. He had been vaccinated six months prior to testing but no serology had been performed at the time. The other, Vietnamese strain 11/144, had leucine instead of methionine at position 133.

The S-amplicon spans nt 497–664 overlying the spacer region of the polymerase gene. There were six strains that were negative by pre-C PCR but positive by the S-gene amplification, and two further strains yielded weak PCR signals and proved unsuitable for sequencing. Sequencing of the distal-X pre-C product in the remaining 50 strains showed that the epsilon (nt 1850–1903) and DR segments (nt 1824–1834) of the amplicon were highly conserved. (Fig. 3). There were two classical pre-C mutants among the 9 HBeAg-negative samples. Only one (V/CS1930) of the other seven yielded a product with our primers and the only nucleotide substitution in its sequence was at nt position 1857 and this T→C change was present in seven other strains. The variability of the distal-X pre-C region was one-third greater than that of the S-region, even though it contains several regulatory sequences (the basic core promoter and enhancer II, as well as the core upstream regulatory region). Thirteen of the 113 nt substitutions encoded amino acid changes in the X-gene. No difference in sequence in either the S or the distal-X pre-C region was found between the first and second samples from any of the 14 index children retested after a two-year interval.

When the relatedness between strains was assessed by comparison of the S-gene sequences from all our subjects, striking clustering was found for strains from particular countries and even closer relationship between strains from members of the same family (Fig. 4). Within genotype B, the predominantly Vietnamese samples could be grouped into three dominant strains. The distal-X pre-C sequence analysis failed to reveal dominant strains, but otherwise showed almost identical grouping of samples into geographical and family clusters (Fig. 5).

DISCUSSION

The introduction of PCR into clinical diagnosis of HBV has stimulated renewed interest in the heterogeneity of HBV genomes both as a determinant of pathogenicity and as a marker of transmission. In this study, all of the subjects were asymptomatic carriers and it is significant that both pre-C and vaccine "escape" mutants were found in this population. This suggests that attribution of a causative effect to mutants detected in patients with active disease or following exogenous immune pressure needs to be supported by studies of the prevalence of such mutants in healthy carriers. The detection of even a single escape mutant among this relatively small sample of strains from a poorly vaccinated population group suggests that laboratory surveillance of vaccination efficacy is warranted, since it is improbable that transmission of such variants to vaccinees would be clinically evident in the short term.

Comparison of phenotypic subtyping with genetic analysis shows excellent correlation between the phenotype inferred on the basis of reported single nucleotide differences and antigenic analysis using monoclonal antibodies. In general, the subtype prevalence in strains from different geographical areas corresponded to reports in the literature. The difference between the dominance of *adr* in Cambodia and Laos and the mixture of *ayw* and *adw* strains in neighboring Vietnam is, however, particularly interesting in a historical context.

Grouping into genotypes offers another means of classifying strains from different sources. The scheme, devised by Norder et al. [1992], is based on dendrograms derived from sequences of 27 HBV genomes, and has substantial correlation with antigenic subtypes, except for the inclusion of both *ayw* and *adw* within genotype B—to which the Vietnamese strains from this

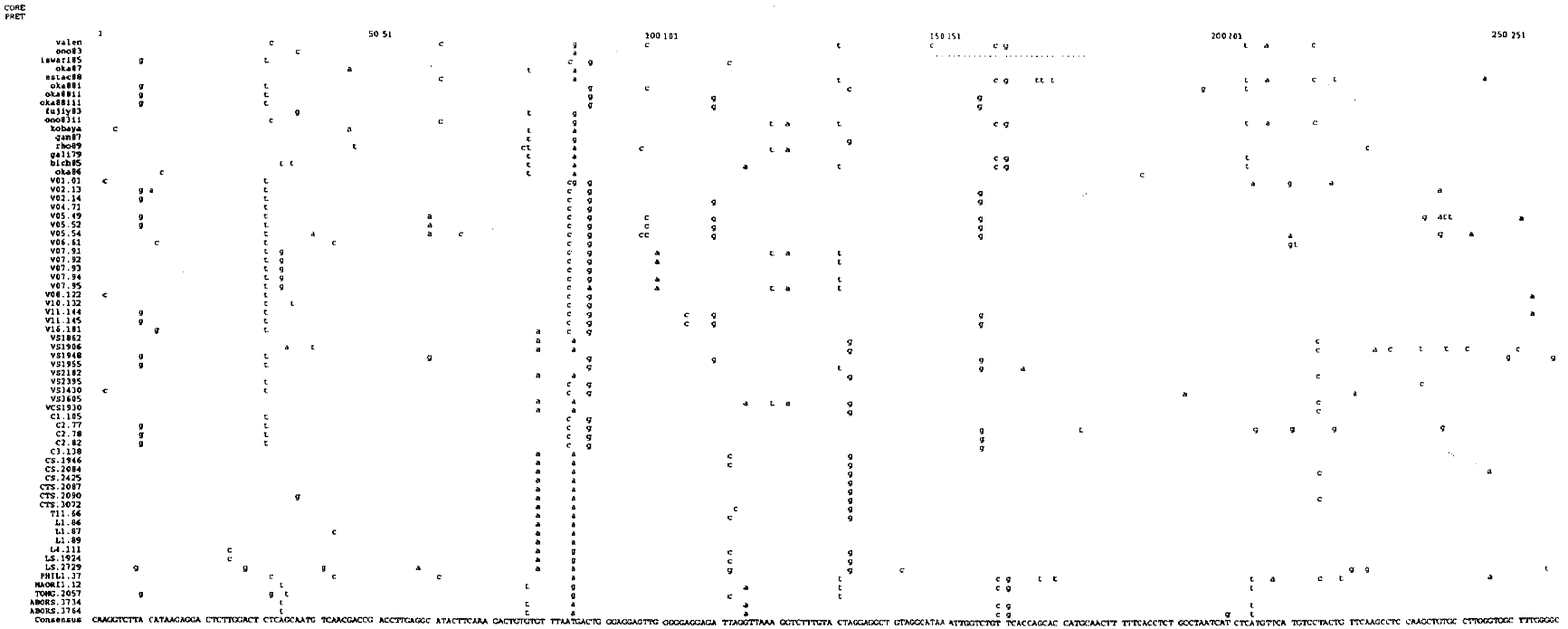

Fig. 3. Nucleotide sequences spanning nt 1643–1900 from the distal-X pre-C region of the 50 study samples, which yielded interpretable data, and from 16 prototype HBV strains. The identification codes for the study samples are shown in Table I.

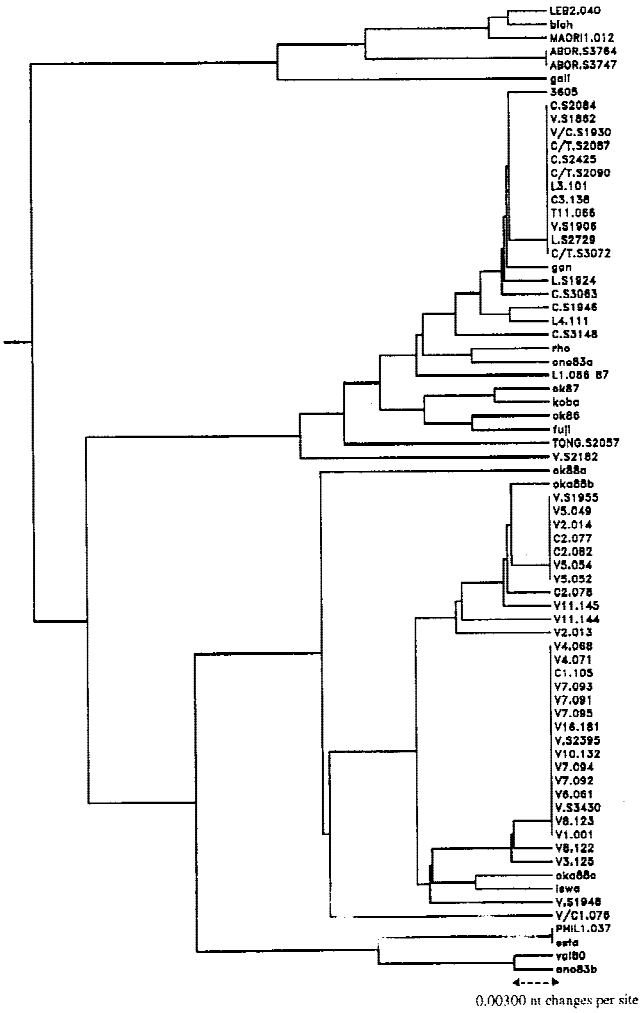

Fig. 4. Dendrogram showing relationships between study samples and prototype strains based on the S-gene nucleotide sequence data. The strain identification codes are shown in Table I.

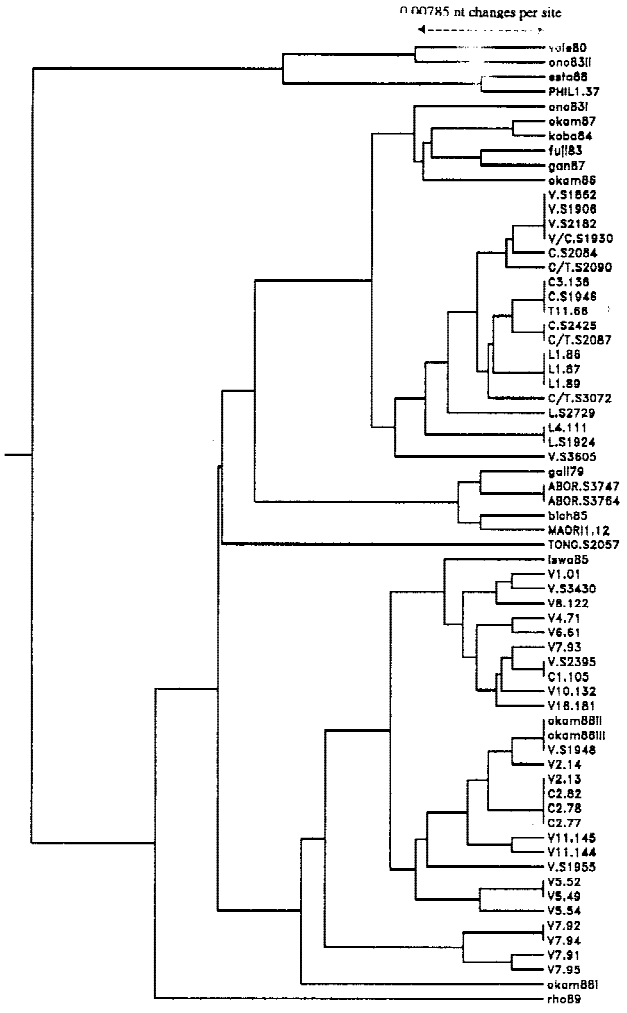

Fig. 5. Dendrogram showing the relationships between study samples and prototype strains based on the distal-X pre-C nucleotide sequence data. The strain identification codes are shown in Table I.

study belong. As more sequence data accumulate from each geographical region, it is to be expected that the genotyping scheme will undergo refinement. In our material, three dominant strains could be discerned from the S-gene sequences belonging to genotype B (Fig. 4), and more extended studies in wider geographical contexts are likely to contribute to finer mapping of these relationships.

Neither classical subtyping nor genotyping is applicable to epidemiological tracing of transmission because of their dominance in particular communities, but the finer divisions that can be achieved by sequence analysis can be used for this purpose. Construction of phylogenetic trees using data from the two different regions of the HBV genome gave almost identical patterns with clear separation of strains from different countries and tight clustering of strains from families within these groups. In two of the three instances where there were differences between stains from different family members (the parents in one family and two adolescent brothers in another), it seems epidemiologically likely that infection was derived from independent sources. This interpretation is supported by the absence of any mutations in the 14 subjects retested after a two-year interval.

For most purposes, data from a single region of the HBV genome appears to be satisfactory, but the failure to amplify the distal-X pre-C region in six S PCR-positive cases may imply the presence of deletion mutants comparable to those described in immunosuppressed carriers by Gunther et al. [1995].

The biological importance of individual mutations is likely to be greatest if amino acid changes in the gene product is affected. Five of the 24 mutations in the 71 S-gene products resulted in amino acid substitution, only one of which corresponded to one of the classical "escape" mutants described by Carman et al. [1990]. Thirteen of the 113 mutations in the 50 distal-X pre-C region were associated with amino acid substitutions. Four of these, including the two 1896 pre-C mutants, lie in the pre-C domain, but the great majority are located in the distal region of the X-protein sequence

where their functional impact is unknown. Deletion of the entire X-gene is compatible with HBV replication [Moriyama, 1997] so it is not surprising to find much greater variability in this region than in the S region where restraint is imposed by the overlapping of gene sequences in different reading frames.

It is concluded that sequence analysis of PCR products, particularly in the hypervariable distal-X region, provides a useful tool for tracing transmission, for instance in suspected point source outbreaks as described by Roll et al. [1995]. The stability of sequence in asymptomatic carriers over time contrasts with reported mutation rates in patients with active liver disease [Blum, 1993]. This may imply that the host maintains active selection of a particular virus population during the carrier phase of hepatitis B, a concept consistent with observations of rapid evolution of HBV sequences in immunosuppressed individuals [Fang et al., 1993].

In terms of the global epidemiology of hepatitis B, construction of dendrograms on the basis of sequence data from short PCR products reveals subfamilies of virus strains within the presently accepted subtyping and genotyping systems. As further data accumulate, these may provide a means of tracing the interaction between HBV and different communities within larger geographical regions.

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